Award Number:
W81XWH-14-1-0195

TITLE:
Novel Mechanism for Reducing Acute and Chronic Neurodegeneration after Traumatic Brain Injury

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CONTRACTING ORGANIZATION:

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REPORT DATE: July 2017

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                          Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2017	Annual	1 Jul 2016 – 30 Jun 2017

4. TITLE AND SUBTITLE

Novel Mechanism for Reducing Acute and Chronic Neurodegeneration After Traumatic Brain Injury

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0195

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Purpose: The purpose of this project is to develop a radically different strategy to reduce brain glutamate excitotoxicity and treat TBI. We will supplement a natural blood-resident enzymatic system with glutamate-oxaloacetate transaminase (rGOT) and the co-substrate oxalo-acetate (OxAc) with the objective of reducing blood levels of glutamate. This will produce a brain-to-blood gradient of glutamate which will enhance the removal of excess glutamate from the brain.
Scope: We will test this novel and powerful neuroprotective treatment in a rat model of repetitive mild (concussive) TBIs and in a model of a single moderate TBI.
Major Findings: We have:
1. Troubleshooting and refinement of blood serum glutamate assays to reduce variability.
2. Completed statistical analysis of behavioral experiments examining effects of rGOT and rGOT + OxAc on outcome on rotarod and Morris water maze.
3. Measured time course of GOT levels in blood and levels after iv injection of 130ug/kg of rGOT.
4. Completed sectioning of brain tissue and completed 60% of hippocampal neuronal cell counting.
5. Troubleshooting and refinement of CSF extraction resulting in reliable measurement of glutamate in CSF.

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	17	19b. TELEPHONE NUMBER *(include area code)*
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Table of Contents

Introduction:

Traumatic brain injury (TBI) continues to be a major problem and has affected hundreds of thousands of service personnel who have served in the Mideast war theater. Many of these personnel have sustained repeated mild or concussive brain injury and now suffer from long-lasting cognitive and physical symptoms. We have developed a radically different strategy to reduce brain glutamate excitotoxicity and treat TBI. We will supplement a natural blood-resident enzymatic system with glutamate-oxaloacetate transaminase (rGOT) and the co-substrate oxalo-acetate (OxAc) with the objective of reducing blood levels of glutamate. This will produce a brain-to-blood gradient of glutamate which will enhance the removal of excess glutamate from the brain. We will test this novel and powerful neuroprotective treatment in a rat model of a single moderate TBI and in a rat model of repetitive mild (concussive) TBIs. Outcome measures include blood and CSF levels of glutamate, acute neuronal degeneration, chronic neuronal cell loss and glial activation. The objective of this project is to evaluate a novel treatment strategy for reducing excessive free glutamate associated with TBI.

This project uses a highly innovative approach to address the long-recognized problem of glutamate excitotoxicity associated with TBI. This novel approach supplements a natural enzymatic system that transforms blood-borne glutamate into α-ketoglutarate. By significantly reducing blood levels of glutamate, a brain-to-blood gradient is produced that enhances the efficiency of Na^+-dependent glutamate transporters located on brain endothelial cells. Thus, excess glutamate in the brain is transported into blood. Compared to the more traditional methods of reducing glutamate excitotoxicity, treatment with rGOT and OxAc circumvents the problems of unwanted side-effect of glutamate antagonists and poor blood-brain barrier penetration associated with receptor antagonist treatments.

Keywords:
Traumatic Brain Injury, Glutamate, GOT enzyme, Oxaloacetate, Fluid percussion, Morris water maze, Rotarod, Behavior

Accomplishments:

What were the major goals of the project?

The major goals of this project are to address the following series of related hypotheses.

- Intravenous administration of rGOT will significantly reduce the concentration of glutamate in blood and subsequently in CSF after TBI
- Treatment with rGOT will reduce functional deficits associated with TBI
- Treatment with rGOT will reduce neural and glial pathology associated with TBI

What was accomplished under these goals?

1. Measured the effects of optimal dose of rGOT and optimal combination dose of rGOT + OxAc on chronic histology on tissue from animals subjected to single moderate TBI.

2. Performed Analysis of blood serum levels of GOT enzyme and pharmacokinetics.

3. Measurement of CSF Glutamate Concentration.

4. Initiated behavioral studies the effects of repeated combination treatments of rGOT and rGOT + OxAc in our model multiple mild TBIs.

Effects of rGOT and combination dose of rGOT + OxAc and repeated combination treatments on chronic histology on animals subjected to single moderate TBI.

We completed this task. We have sectioned and mounted all tissue for histological staining for the four groups listed in Table 1. We have completed staining of the tissue with cresyl violet and have completed stereological cell counts of surviving neurons in the hippocampus. Results are shown in graph of Figure 1.

Anatomical Regions of Interest and Stereological Cell Counting Methods

The region of interest for measurement of surviving neuronal cells encompassed the dorsal stratum pyramidale of the hippocampus CA2 and CA3 (Figure 2B). Systematic random sampling techniques were used for selecting tissue sections for staining and stereological analysis. Every fifth section was sampled starting at a section randomly determined from the first through fifth rostral-most sections. The tissue sections were then mounted onto gelatin-coated slides and stained with the Nissl stain, cresyl violet.

Pyramidal neurons counts of the CA2 and CA3 hippocampal fields were performed by an investigator uninformed of the group assignment. Sections were examined on a microscope with a motorized stage using computer software (Stereo Investigator). The region of interest was outlined under 4X magnification (Figure 2B). Criterion for counting pyramidal neurons required visualization of the nucleus of morphologically distinct cell bodies. Neuronal cell counting was performed with a 100X oil objective (Figure 2C,D). The total number of neurons in the region of interest was quantified using optical fractionator stereological methods. The spacing of the optical dissectors produced an average area sampling fraction (ASF) of 0.030. The guard height was set at 0.40 μM producing a tissue sampling fraction (TSF) of 0.70. Target cells in every fifth section were counted producing a section sampling fraction (SSF) of 0.20.

Final Results

We performed ANOVA followed by post hoc Tukey HSD. The overall ANOVA was significant $F(3,27)=17.92$, $p<0.001$ indicating differences between groups. The post hoc Tukey test indicated that TBI with vehicle treatment produced a significant loss of ~ 21,600 CA2/3 pyramidal neurons compared to sham injury. Treatment with rGOT alone did not affect neuronal cell counts compared to the TBI vehicle-treated group. There was a trend for the rGOT + Oxaloacetate -treated group to have an increased number of neurons (~7,000) compared to the TBI Vehicle group (p=0.089).

Groups	Weight (g)	ATM
Sham (n=5)	333 ± 17	n/a
TBI + Vehicle (n=10)	340 ± 21	2.13 ± 0.01
TBI + rGOT (n=8)	339 ± 18	2.15 ± 0.01
TBI + rGOT + Oxal (n=8)	326 ± 21	2.15 ± 0.02

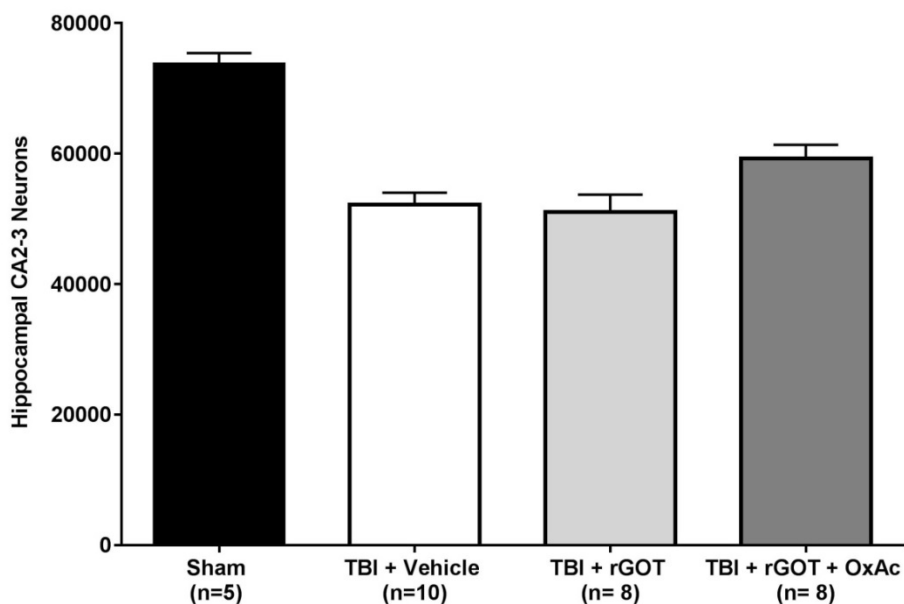Table 1. Groups, Sample size, Body weight, ATM (means ± SD)

Figure 1: CA 2/3 neuronal cell counts performed at day 14 post-injury. TBI produced a significant loss of neurons. Treatment with rGOT + OxAc produced a trend for increased survival of neurons compared to the TBI vehicle group.
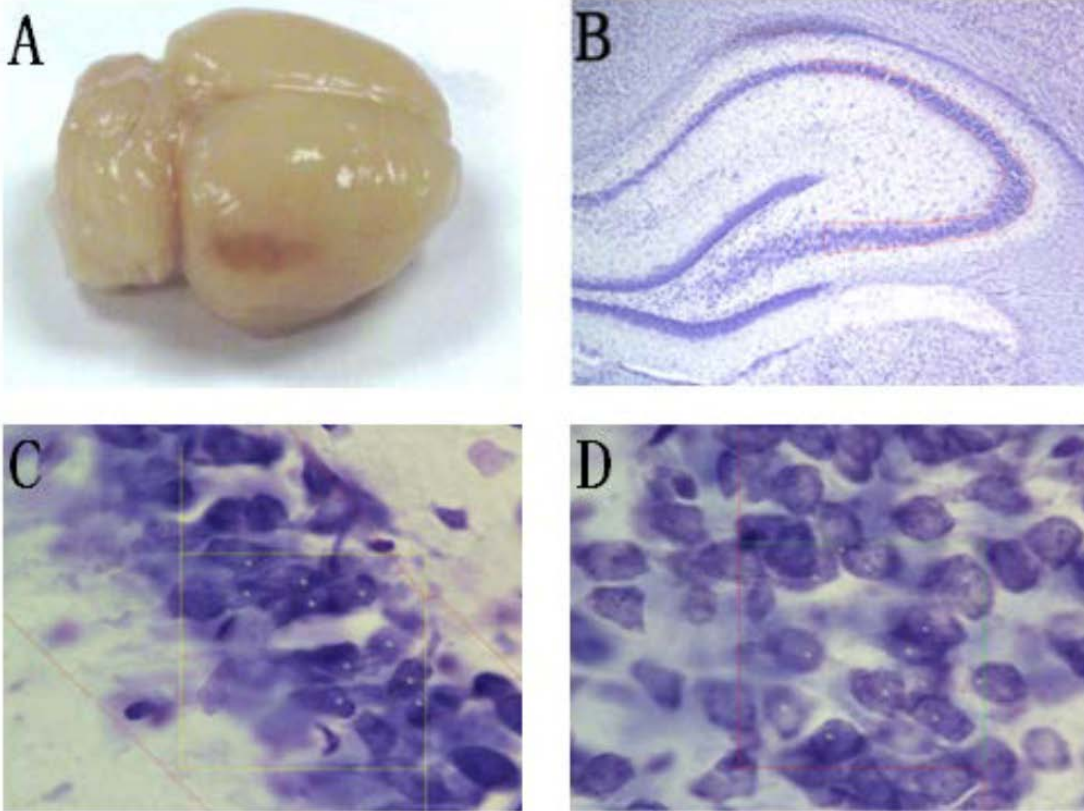
Figure 2: Examples of TBI brain at 14 days post-injury. (A): Gross pathology. Note typical area of infarction in the ipsilateral parietal cortex.   (B): Coronal section of ipsilateral hippocampus stained with cresyl violet (4X magnification)   (C): Representative section from the ipsilateral CA2 (100X oil)   (D): Representative section from the ipsilateral CA3 (100X oil)

Analysis of blood serum levels of GOT enzyme

We have performed analysis of GOT-1 enzyme levels in blood serum with our moderate and multiple mild TBI paradigms.

Blood samples were obtained via tail vein prior to moderate TBI and at 24 hours after TBI. For multiple mild TBIs, tail vein blood samples were taken prior to the first bilateral mild TBI, 1 hour post TBI and 24 hour post TBI (which was just prior to the second bilateral TBI), one hour post the second bilateral TBI and finally at 24 hours post the second bilateral TBI.

Blood samples were coagulated and centrifuged to produce serum samples. Samples were analyzed at the UC Davis Comparative Pathology Laboratory using a Roche Diagnostics Cobas Integra 400 Plus clinical chemistry analyzer.

The multiple mild bilateral TBIs: Rats were mounted in a stereotaxic frame, a scalp incision made along the midline, two 4.8-mm diameter craniectomies were performed on the right and left parietal bone (centered at -4.5 mm Bregma and right and left lateral 3.0 mm). A rigid plastic injury tube (modified Leur-loc needle hub, 2.6-mm inside diameter) was secured to each of the craniectomies with cyanoacrylate adhesive. Care was taken to leave the exposed dura intact. Two skull screws (2.1 mm diameter, 6.0 mm length) were placed into burr holes, 1 mm rostral to Bregma and 1 mm caudal to Lambda.

A fluid percussion pulse of 1.25 ATM to create a mild TBI was delivered to the right hemisphere after disconnection from the ventilator and then repeated on the contralateral side one minute later. During each percussion pulse, the contralateral non-pulsed injury tube was plugged to prevent leakage of saline. Immediately after the bilateral TBIs were induced, the rat was ventilated with a 2:1 nitrous oxide/oxygen mixture. The two injury tubes were then plugged, and the scalp was sutured. The repetitive aspect of the injury was performed twenty-four hours later.

Results show that TBI produced an increase in endogenous serum GOT measured at 1 hour after a single moderate TBI (Figure 3). This was also observed after a single bilateral mild TBI (Figure 3). In our multiple bilateral mild TBI paradigm, GOT levels in blood serum were elevated after the first bilateral TBI at 1 hour and then returned to baseline at 24 hours and was again elevated to a similar level at 1 hour after the second bilateral mild TBI - returning to baseline 24 hours later (Figure 4).

We speculate that this elevation may be due to systemic effects of the brain injury on organ function, notably liver function where GOT is produced. Acute sympathetic nervous system activation may contribute to elicit both inflammation and immunodepression This may be occurring through multiple pathways including (1) tissue chemokines producing inflammation in peripheral organs, (2) activation of monocytes and IL-10, (3) HPA activation. Interestingly, this increased GOT level response is similar in mild and moderate TBI and was observed after the first and second bilateral mild TBI events.

It is important to note that the beneficial effects of administering exogenous rGOT enzyme greatly increases the serum levels of GOT (Figure 5) far in excess (15-fold difference) of the elevation we have shown in Figure 3 due to the TBI alone.
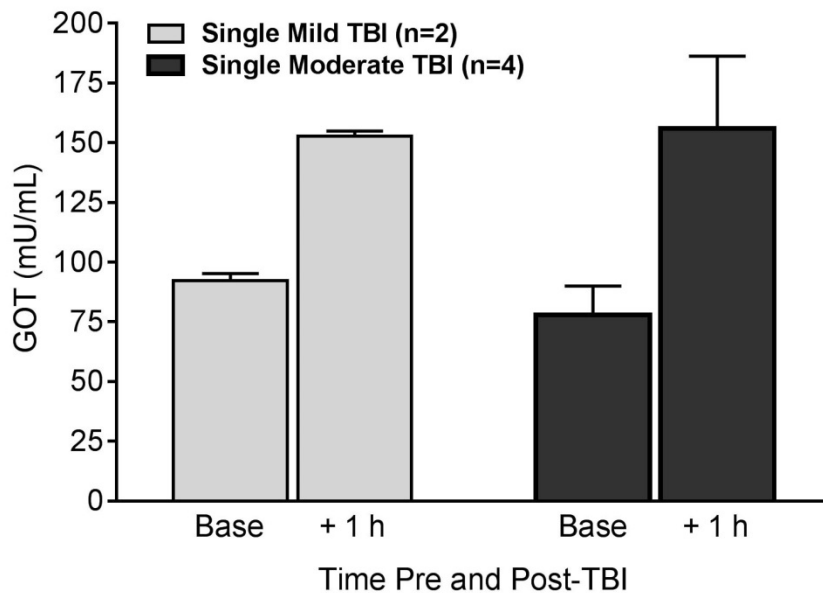

Figure 3. Endogenous blood serum levels of GOT enzyme increase 1 hour after a single mild bilateral TBI or after a single moderate lateral TBI.
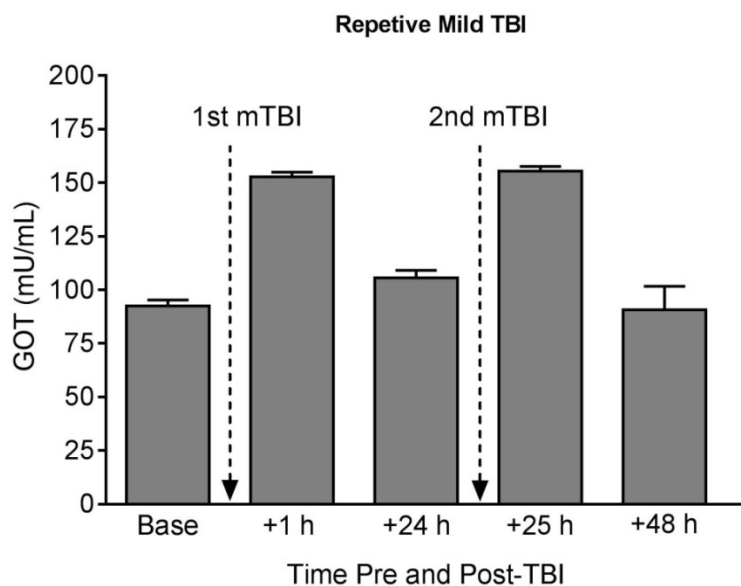

Figure 4. Endogenous blood serum levels of GOT enzyme increase 1 hour after mild bilateral TBI and return to baseline by 24 hours after TBI (n=4). This pattern is nearly identical after a second mild bilateral TBI.
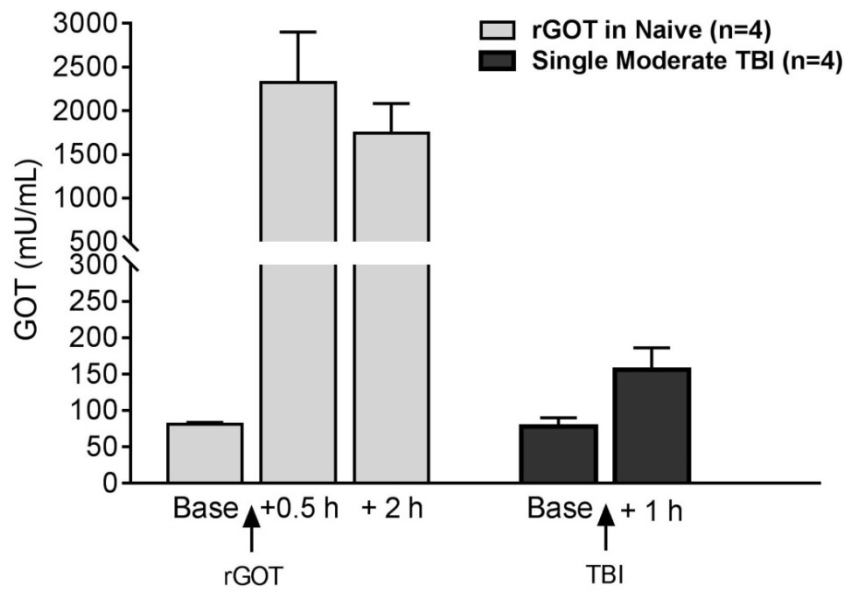
9

Figure 5. Comparison of blood serum levels of GOT enzyme after i.v. administration of exogenous rGOT enzyme (light grey bars) and after moderate TBI (black bars). Note that the elevation on blood serum levels of GOT enzyme are 15-fold higher after the iv injection of rGOT versus the blood serum levels of GOT at 1 hour after TBI.
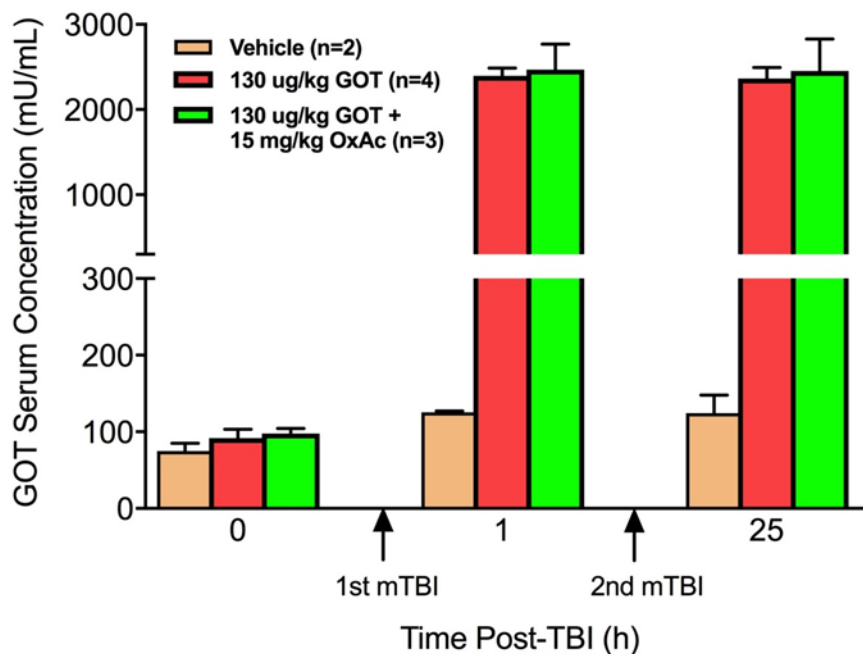

Figure 6. Comparison of blood serum levels of GOT enzyme after i.v. administration of exogenous rGOT enzyme after first and second mild TBI. Note the consistent elevation of serum GOT after each mTBI (rGOT was injected 5 minutes after each mTBI.
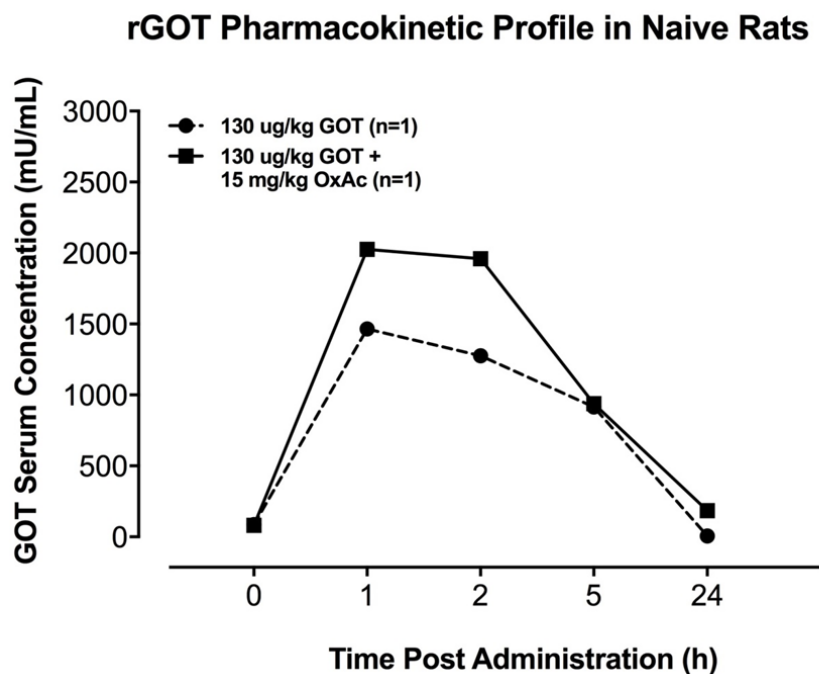
Figure 7. Pharmacokinetic profile of serum levels of GOT following a single rGOT or rGOT + OxAx injection. Blood serum levels of remain highly elevated for at least 2 hours after injection and began to decline by 5 hours post-TBI.

Measurement of CSF Glutamate Concentration.

We have begun sampling CSF from cisterna magna and performing analysis of glutamate content. Figures 6 shows a standard curve and Table 2 and Figure 7 show results of five naïve animals in which CSF and blood serum were collected and analyzed with our colorimetric glutamate analysis kit. We detected lower levels of glutamate in the CSF as compared to plasma.
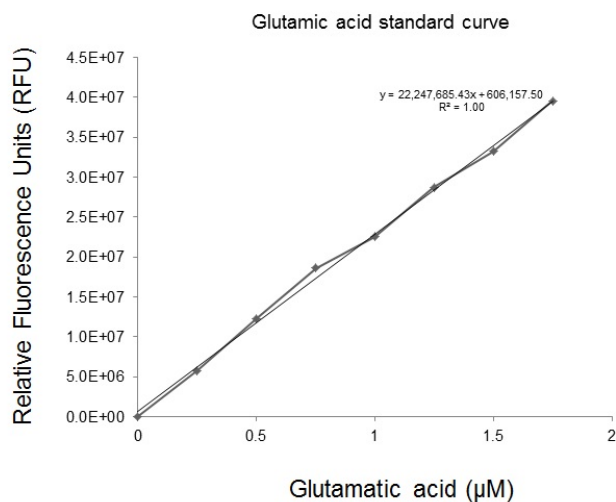

Figure 6. Glutamate standard curve.

11

We have solved our technical problems of sampling CSF from the cisterna magna and have now performed analysis of glutamate content in CSF and serum from 5 naïve rats. Table 2 shows individual data from each rat. Figure 7 shows average results of five naïve animals in which CSF and blood serum were collected and analyzed with our colorimetric glutamate analysis kit. As predicted, we detected much lower levels of glutamate in the CSF as compared to plasma. CSF glutamate levels are now similar to those reported in the literature.

Treatment Group	Weight (g)	Glutamate Levels in CSF (µM)	Glutamate Levels in CSF (µM)
Naïve- rat #1	323	5.78	116
Naïve- rat #2	352	3.66	93.6
Naïve- rat #3	343	9.67	91.9
Naïve- rat #4	355	7.08	93.8
Naïve- rat #5	327	8.03	112.7
Mean ± SD	340 ± 14.5	6.84 ± 2.28	102 ± 11.7

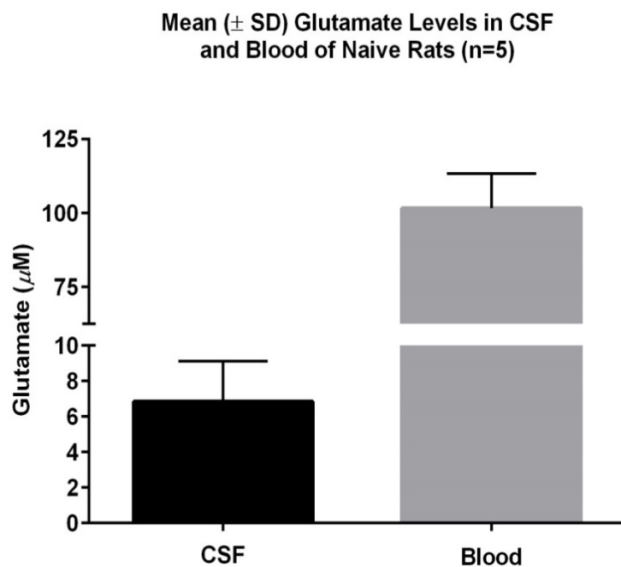**Table 2.** Glutamate Levels in CSF and Blood from Naïve Rats.

Figure 7   Comparison of CSF and serum glutamate levels in naïve rats (mean + SD).

Effects of optimal dose of rGOT and optimal combination dose of rGOT + OxAc with repeated treatments on motor behavioral and cognitive performance after multiple mild TBIs.

We are in process of evaluating the effects of rGOT treatment in our model of multiple mild TBIs. Outcome measures include cognitive and motor performance through day 14 post-injury. Current accrual is: Vehicle (n=2), rGOT (n=4), rGOT + OxAc (n=3).

What opportunities for training and professional development has the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
Preliminary results were presented in an oral session at the 2015 MHSRS Symposium.

What do you plan to do during the next reporting period to accomplish the goals?
During quarter one of year four (no-cost extension), we will proceed to complete the studies of multiple mild TBIs.

Reportable Outcomes:
Published manuscript: Lyeth BG (2016) Historical Review of the Fluid Percussion TBI Model. Frontiers Neurology 7:217. Doi:10.3389/fneur.2016.00217

Impact:

What was the impact on the development of the principal discipline(s) of the project?
 The results thus far on this project have a potentially high impact on the acute treatment of moderate TBI. The significant reduction in motor and cognitive deficits using a treatment that does not have to penetrate into the brain parenchyma is quite remarkable. The rGOT treatment targeting glutamate excitotoxicity is especially noteworthy since so far there are no indications of toxic or adverse effects.

What was the impact on other disciplines?
 The positive results of the rGOT treatment can have an impact on other neurological disorders that are mediated by glutamate excitotoxicity such as stroke and epilepsy.

What was the impact on technology transfer?
 The rGOT technology devised by collaborator Dr. Mirelman at the Weizmann Institute may have a substantial impact upon future treatments of TBI and other conditions involving glutamate excitotoxicity.

What was the impact on society beyond science and technology?
 Nothing to Report

Changes/Problems
 We experienced a slowdown in performance due technician Ken Van having to take extended family leave to care for a critically ill infant. These issues have resolved and we expect performance to accelerate during year four.

Products:
 Nothing to Report

Participants & Other Collaborating Organizations:
 What individuals have worked on the project?

Name:	Bruce Lyeth, PhD
Project Role:	Principle Investigator
Researcher Identifier:	252972781 (UC Davis ID)
Nearest person month:	2
Contribution to the project:	Dr. Lyeth performed the fluid percussion TBIs and supervised the conduct of the project.
Funding Support:	1R01NS089901, NIH (PI: Liu, Dazhi) 20% effort; 1R43DA041760-01, NIH (PI: Fitzpatrick, B.) 7% effort

Name:	Ken Van, MS
Project Role:	Staff Research Associate
Researcher Identifier:	613144013 (UC Davis ID)
Nearest person month:	6
Contribution to the project:	Mr. Van performed the surgeries, the blood draws, glutamate assays, and behavioral testing.

Funding Support:	No other source

Name:	Gene Gurkoff, PhD
Project Role:	Assistant Researcher
Researcher Identifier:	727993875 (UC Davis ID)
Nearest person month:	1
Contribution to the project:	Dr. Gurkoff assisted with the serum glutamate assays.
Funding Support:	No other source

Name:	Emily Doisy, BS
Project Role:	Staff Research Associate
Researcher Identifier:	897554960 (UC Davis ID)
Nearest person month:	1
Contribution to the project:	Ms. Doisy is the Laboratory Manager & Safety Officer. She managed ordering of supplies and managed safety training and concerns in the laboratory. She also assisted with the serum glutamate assays.
Funding Support:	No other source

Has there been any changes in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
No changes in support for the PI.

What other organizations were involved as partners?
Collaborator, Dr. David Mirelman of the Weizmann Institute was instrumental in providing rGOT for this project. He also participated in monthly discussion via Skype and with annual visits to UC Davis to discuss scientific progress and problems.

Special Reporting Requirements:
  Collaborative Awards:
Not applicable

  Quad Chart:
Updated quad chart is attached.

Appendices:
None

15

Novel Mechanism for Reducing Acute and Chronic Neurodegeneration after TBI

Log number: PT120075
Award Number: W81XWH-14-1-0195

PI: Bruce Lyeth, Ph.D.   **Org: University of California, Davis**   **Award Amount : $763,916**

DMRDP

Study Aims

- **Specific Aim 1:** Determine the effects of TBI on glutamate levels in serum and CSF after TBI and determine the effects of rGOT on glutamate after multiple mild or a single moderate TBI in rats.
- **Specific Aim 2:** Determine the effects of the optimal doses of rGOT on acute and chronic brain pathology and behavioral outcome after TBI in rats.
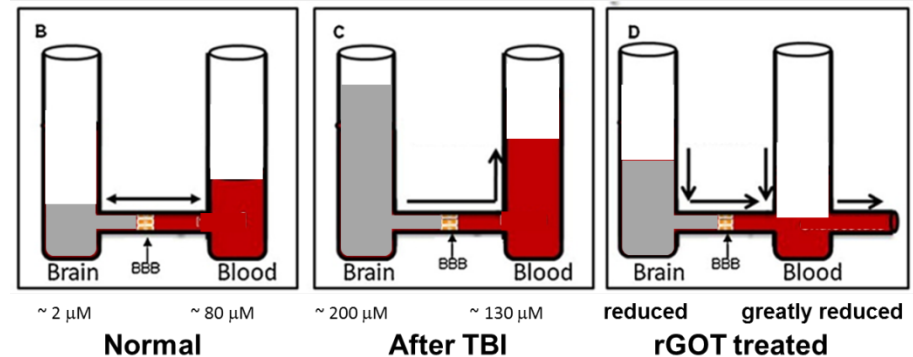
Approach

An intravenous administration of the enzyme, glutamate oxaloacetate transaminase (GOT) that converts glutamate into α-ketoglutarate will be evaluated for reducing excessive free glutamate associated with TBI. We will examine the mechanism of action of GOT on blood and CSF levels of glutamate and the therapeutic potential of GOT to reduce cellular and behavioral pathology associated with TBI. These objectives are addressed using two clinically relevant models of experimental TBI in the rat.

Mechanism of glutamate diffusion between blood and brain:
rGOT treatment reduces blood glutamate, creating a diffusion gradient that scavenges glutamate from the brain.

Estimated Glutamate Concentration

	Brain	Blood
Normal	~ 2 µM	~ 80 µM
After TBI	~ 200 µM	~ 130 µM
rGOT treated	reduced	greatly reduced

Timeline and Cost

Activities FY	14	15	16	17
Aim 1a: Determine optimal rGOT dosing for reducing blood and brain levels of glutamate.	▅			
Aim 1b: Determine effects of rGOT on chronic pathology and behavior after repeated mild TBI				▅
Aim 2: Determine effects of rGOT on pathology and behavior after moderate TBI		▅		
Estimated Budget: $K in total costs	254	254	254	EWOF

Goals/Milestones

CY14 Goal – Experiment ramp-up & determine optimal dosing
- ☑ Optimize experimental methods and glutamate assay
- ☑ Determine optimal rGOT dosing for reducing glutamate in blood

CY15 Goals – Evaluate treatment effects on moderate TBI
- ☑ Determine rGOT effects on brain pathology after moderate TBI
- ☑ Determine rGOT effects on behavior after moderate TBI

CY16 Goal – Evaluate treatment effects on repeated mild TBI
- ☐ Determine rGOT effects on behavior after mild TBI
- ☐ Determine rGOT effects on brain pathology after mild TBI

Comments/Challenges/Issues/Concerns

- CY16 goals to be completed during 1-year EWOF.
- spending is well below projections and will allow sufficient funds for EWOF.

Budget Expenditure to Date

Projected Expenditure: $763,916
Actual Expenditure: $681,554

Updated: 25 JUL 2017